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PRINCIPAL INVESTIGATOR: Roshan Karunamuni

CONTRACTING ORGANIZATION: University of Pennsylvania
Philadelphia, PA 19104

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Roshan Karunamuni
Email: aros@seas.upenn.edu

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14. ABSTRACT
Targeted imaging agents use specific biomarkers that are present in tumor tissue to distinguish cancerous cells from their immediate benign environment. They are able to provide both structural and functional characteristics of the tumor such as shape, size, growth rate and expression level of cell-surface makers. The aim of the study was to demonstrate, in proof-of-principle, the applicability of bioconjugated gold (Au) nanoparticles (NP) as targeted contrast agents for use in breast x-ray imaging. I have successfully synthesized AuNP, with an average diameter of 21 nm, that have been surface-stabilized with polyethylene glycol (PEG) chains. The nanoparticles have also been concentrated to an extent where they show an observable radiographic contrast compared to water. I am currently procuring animals to study the *in vivo* characteristics of these surface-stabilized AuNP.

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1. Introduction

Targeted imaging agents use specific biomarkers that are present in tumor tissue to distinguish cancerous cells from their immediate benign environment. They are able to provide both structural and functional characteristics of the tumor such as shape, size, growth rate, and expression level of cell-surface markers. Today, the most commonly used x-ray contrast agents are iodine-based compounds [1]. However, the non-specific nature of these agents results in random vascular permeation, rapid renal clearance, and poor tumor-targeting potential.

This research aims to demonstrate, in proof-of-principle, the applicability of bioconjugated gold (Au) nanoparticles (NP) as targeted contrast agents for use in breast x-ray imaging. Gold is roughly three times more attenuating than iodine at energies between 10 – 32 keV, and would thus be able to provide superior contrast at these energies. The nanoparticles will be functionalized with an anti-HER2/neu targeting ligand to help discriminate between breast tumor cells and normal tissue. HER2/neu is a cell surface receptor protein that is overexpressed in roughly 25-30% of all breast cancers [2, 3]. The combination of such contrast agents with temporal subtraction breast tomosynthesis (DBT) or digital mammography (DM) would allow high-resolution cross-sectional molecular imaging *in vivo* and trivial fusion of functional and anatomic images.

I have, thus far, synthesized and characterized spherical AuNP with an average diameter of 14 nm. Future directions include attachment of a targeting ligand to the surface of the AuNP, as well as evaluating the *in vivo* effect of the nanoparticles.

2. Body

2.1. Research Overview

Over the past year, my effort has been directed towards developing bioconjugated AuNP that could have potential application as targeted imaging agents for breast x-ray imaging modalities, such as DBT or DM.

In order to be used as x-ray targeted imaging agents, the gold nanoparticles must fulfill certain criteria:

- provide sufficient contrast with x-ray imaging modalities
- able to differentiate between tumor cells and normal tissue
- non-toxic, *in vivo*

We have chosen to use gold spherical nanoparticles as the building block for our imaging agents because of their relative ease of synthesis and functionalization. The AuNP were surface stabilized with polyethylene glycol (PEG) chains that have been shown to enhance stealth characteristics and improve longevity of nanoparticles *in vivo*. The physical (diameter, size distribution) and surface (hydrodynamic diameter, zeta potential) characteristics of the AuNP were determined through a combination of transmission electron microscopy (TEM), dynamic light scattering (DLS), and UV/Vis measurements.

The AuNP were imaged using a conventional digital mammography system to quantify the x-ray contrast that they provide.

The research therefore consists of three major subsections:

(i) Synthesize, functionalize and concentrate AuNP
(ii) Characterize the structural and radiographic properties of the AuNP.
(iii) Evaluate the in vivo effect of the nanoparticles: tumor-enhancement, biodistribution, and toxicity

I have, over the course of the year, successfully completed the first two major aims and the results obtained are presented herewith. I have decided to test the properties of AuNP with a PEG coating first, before I investigate AuNP that possess both PEG and anti-HER2/neu ligands. This will allow for us to familiarize ourselves with the necessary techniques for synthesis and characterization of the nanoparticles, while still working towards the development of targeted AuNP . I have also included an outline of the research I plan to pursue in the upcoming year.

2.2. Results

2.2.1. Synthesis of gold nanoparticles

AuNP have been synthesized using a modified Turkevich method, as detailed by Grarbar *et al.* [4]. The method involves the reduction of a boiling solution of gold chlorate by citrate anions, which serve as both the reducing agent and surfactant. The citrate anions form a protective barrier around the AuNPs and prevent separate particles from fusing together. The final size and distribution of the particles obtained is dependent on a multitude of factors including the pH and temperature distribution of the boiling gold solution, but most importantly on the molar ratio between the gold chlorate and citrate solution. Described here are the steps used in the synthesis of AuNP with an expected diameter in the range of 13 nm using a 4:1 ratio between the gold and citrate.

All glassware used in the synthesis was first treated with aqua regia (3 parts nitric acid, 1 part hydrochloric acid), rinsed with deionized (DI) water (H_20) ,and then oven-dried. Briefly, 321.6 mg of gold (III) chloride trihydrate ($HAuCl_4.3H_2O$) was dissolved with 450 mL of DI-H_2O and placed in a 1L round-bottom flask with a Teflon-coated magnetic stirring rod. The flask was fitted with a condenser, placed atop a hearing mantle and magnetic stirrer, and the gold solution was brought to a boil (Figure 1a). Next, 0.93 g of sodium citrate dihydrate ($C_6H_5Na_3O_7.2H_2O$) was dissolved in 45 mL of DI-H_2O and added to the boiling gold solution. Upon addition of the sodium citrate, the solution rapidly changed color from a pale yellow to a deep burgandy. Heating was continued for an additional 10 minutes after which the heating mantle was removed, and the solution was stirred for a further 15 minutes. The resulting colloidal AuNP solution was allowed to cool overnight and then filtered through a 0.2 μm membrane filter.

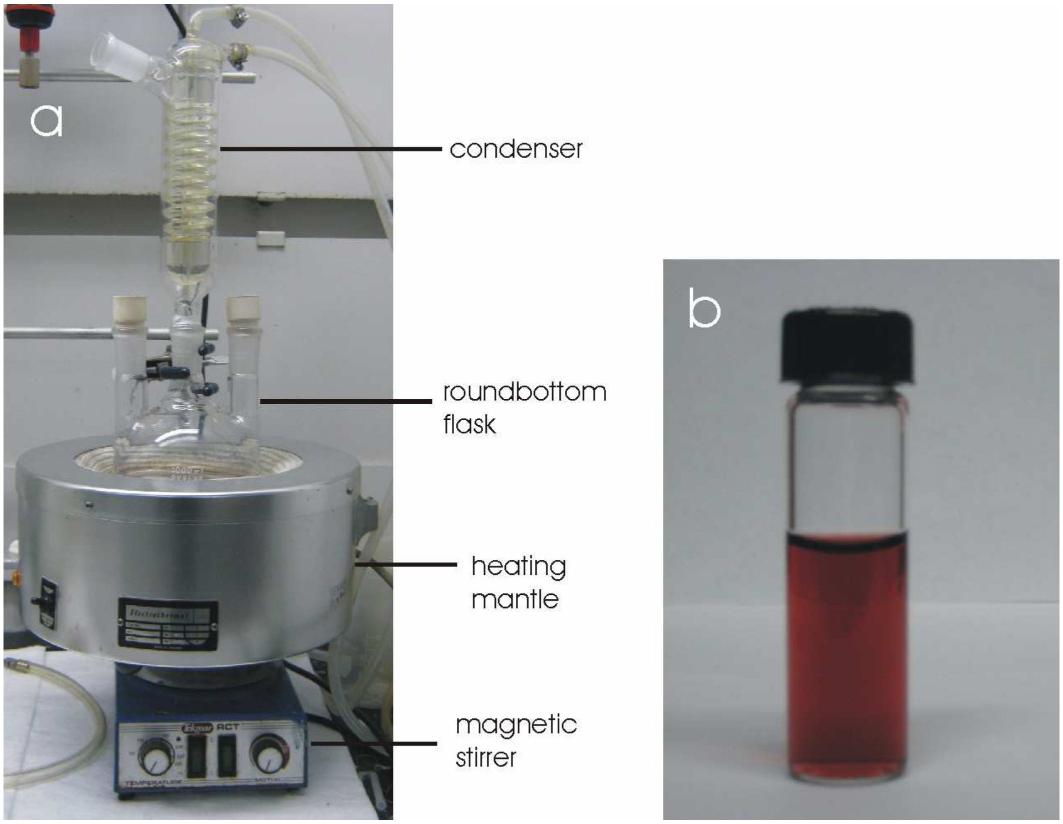

Figure 1. (a) Synthesis Setup (b) citrate-capped AuNP

2.2.2. Functionalization (surface-stabilization) of AuNP with PEG

Citrate-capped AuNP (cAuNP) are however unsuitable for use *in vivo*. The citrate anions that coat the surface of the nanoparticles impart a negative surface charge to the cAuNP which would promote the non-specific attachment of serum proteins *in vivo*. This would severely hinder the ability of the cAuNP to reach the tumor site. The cAuNP are also stabilized through mutual electrostatic repulsion between neighboring negatively-charged nanoparticles. This repulsion can be shielded by ions present in a salt-like, or ionic, solution (ex. blood plasma), which would result in the aggregation of the cAuNP.

The cAuNP were thus conjugated with polyethylene glycol (PEG) chains in order to obtain surface-stabilized AuNP (ssAuNP) that were stable in blood serum, and exhibited enhanced stealth properties *in vivo* [5-7]. The PEG chain chosen for the conjugation has an average molecular weight of 5000 Daltons and possesses an unreactive methyl group on one end, and a thiol (-SH) group for direct attachment to the gold surface on the other (Figure 2). The conjugation scheme was based on a similar procedure by Bergen *et al.* [8], which assumed a 4 PEG/nm^2 surface coverage.

Described here is an example of a typical functionalization procedure. 30 mL of cAuNP was mixed with 6.7 mL of a 272 μM PEG solution in an Erlenmeyer flask, and stirred at room temperature for 1 hour. The solution was then centrifuged twice at 11,400g for 45 minutes, after which the ssAuNP pellet was resuspended in 2 mL of DI-H_2O. The ssAuNP were concentrated further by resuspending the centrifugation pellet in successively smaller volumes.

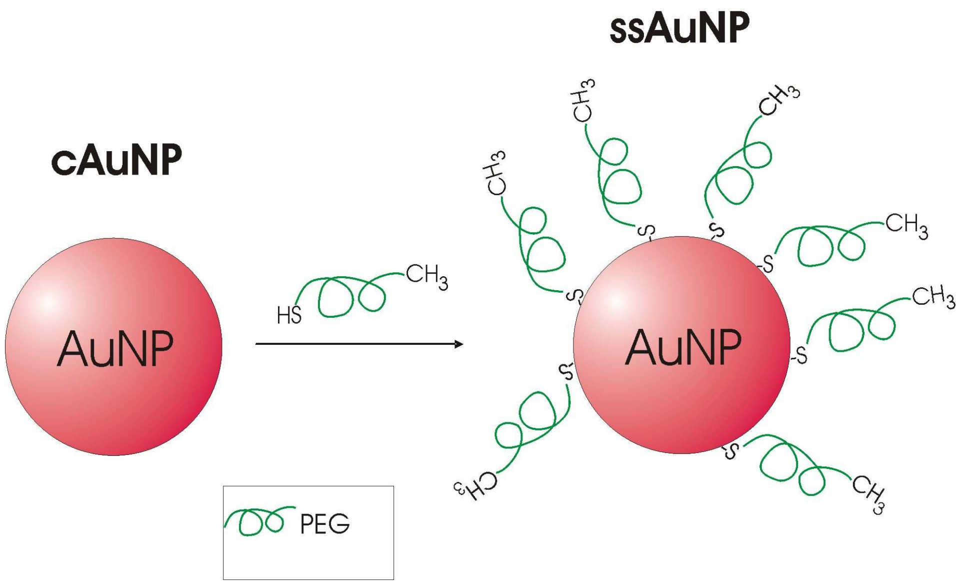

Figure 2. Surface-stabilization scheme of citrate-capped AuNP (cAuNP) with PEG chains to produce surface-stabilized AuNP (ssAuNP)

In order to demonstrate the improved stability that is a result of the surface stabilization of the AuNP with PEG, 800 uL each of cAuNP and ssAuNP were mixed with 400 uL of phosphate-buffered saline (PBS) – a common buffer solution whose osmolarity and ion concentration match those of the human body. As shown in Figure 3, addition of PBS to the cAuNP resulted in a aggregation of the particles and a change in color from red to purple-blue. The ssAuNP ,by contrast, showed no observable change in color or stability after the PBS had been added.

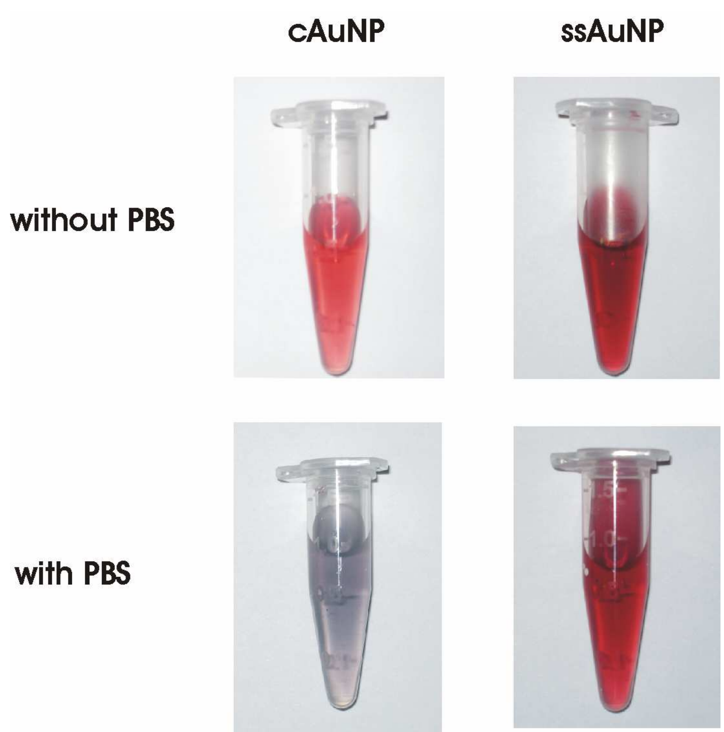

Figure 3. Effect of PBS on cAuNP and ssAuNP.

2.2.3. AuNP Characteristics - TEM

The diameter of the AuNP was determined through TEM. A drop of the AuNP (cAuNP and ssAuNP) solution was placed on a carbon-coated copper grid and allowed to dry for 6 hours prior to imaging. The grid was then imaged using a high-resolution transmission electron microscope, and the images obtained are shown in Figure 4 . The size of the AuNP was determined by individually measuring the diameter of 50 imaged nanoparticles, and averaging the result. The average diameter of the AuNP was found to be 21 nm, with a standard deviation of 4.2 nm.

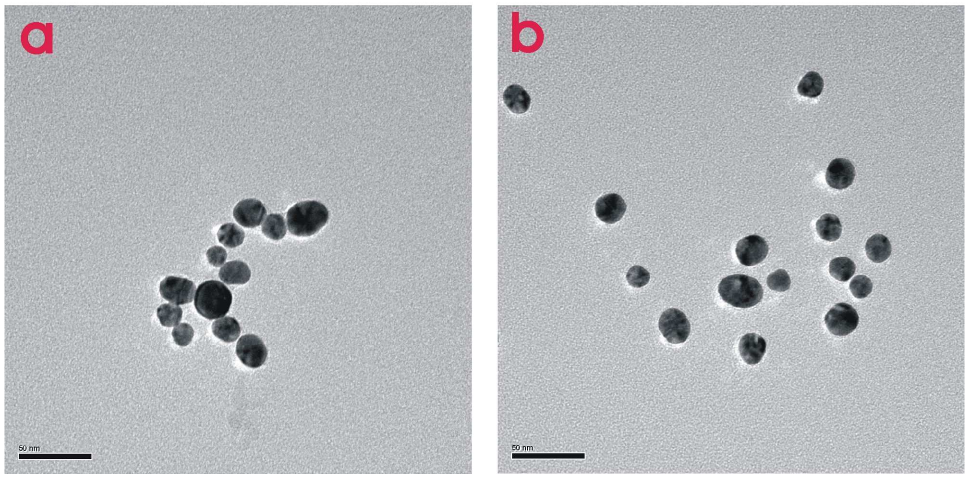

Figure 4. TEM images of (a) cAuNP and (b) ssAuNP. The scale bar in the images corresponds to 50 nm.

The ssAuNP (Figure 4b) appear to be further spaced apart than the cAuNP (Figure 4a) due to the presence of the PEG layer on the surface of the nanoparticles. The PEG chains provide a physical barrier between the nanoparticles, and thus prevent them from coalescing or clumping together.

2.2.4. AuNP Characteristics – UV/Vis Spectra

The absorbance of the AuNP in the ultraviolet (UV) to visible(Vis) range was measured using a Perkin Elmer UV/Vis spectrometer. The particles exhibit a characteristic absorbance peak that is typical of most colloidal metal nanoparticle solutions. This peak is a result of the coherent oscillation of free conduction electrons in nano-sized metal structures in the presence of an electromagentic field; a phenomenon commonly referred to as surface plasmon resonance (SPR). The peak wavelength of the SPR is determined by the size of the nanoparticles, the presence of a surface agent, as well as the pH of the surrounding solution.

Figure 5 shows the SPR of the cAuNP, with a peak wavelength of 522 nm. The concentration of the nanoparticles was determined using extinction coefficients present in the literature [9], and was found to be 20.7 nM. Figure 6 shows that the normalized SPR of the ssAuNP is shifted to the red by 2 nm compared to that of the cAuNP. This shift in the SPR helps confirm the presence of the PEG layer on the surface of the ssAuNP.

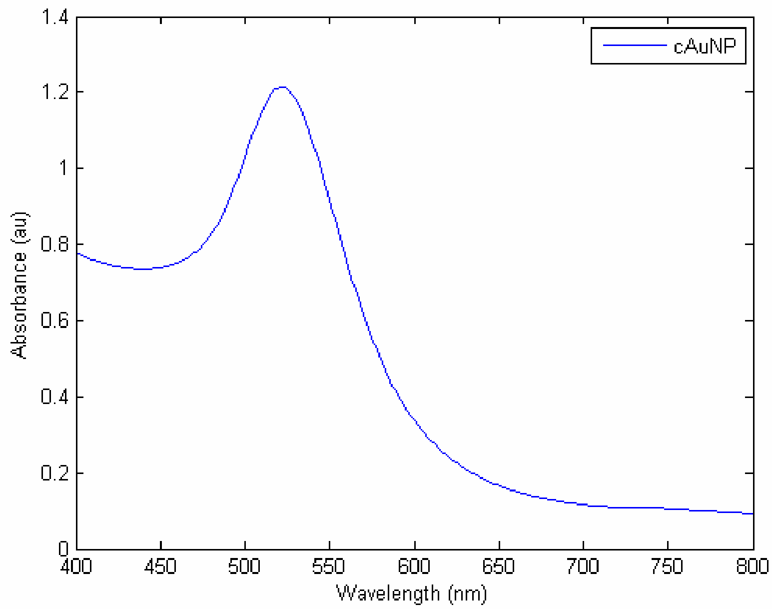

Figure 5. SPR of cAuNP in the UV/Vis range.

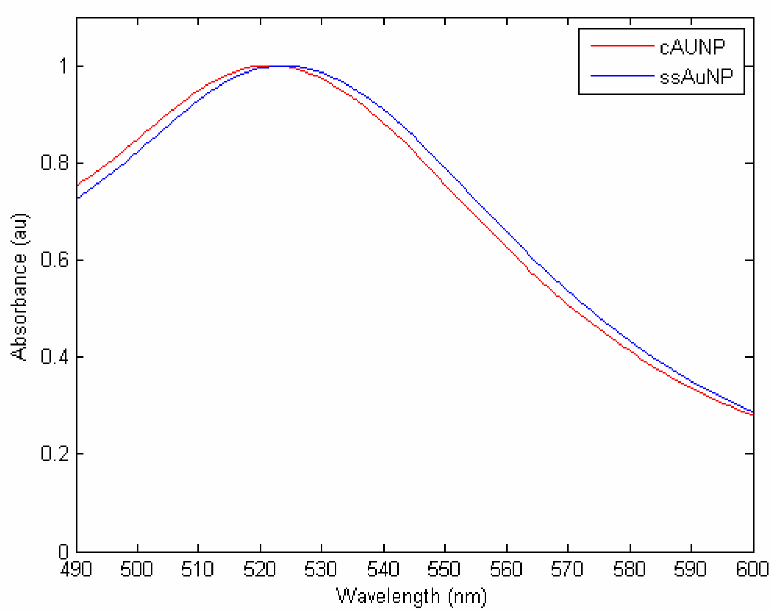

Figure 6. Red-Shift in the normalized SPR of the ssAuNP compared to cAuNP. This finding helps confirm the presence of the PEG layer on the surface of the ssAuNP.

2.2.5. AuNP Characteristics – Zeta Potential

The zeta potential of the AuNP solutions was obtained for various surfactant surface densities (in number of PEG molecules per nm^2). A surface density of 0 PEG/nm^2 corresponds to cAuNP, whereas a density of 4 PEG/nm^2 corresponds to the ssAuNP that have been used previously. Mixtures of AuNP with 1 and 20 PEG/nm^2 were synthesized especially for this experiment. As expected, the cAuNP showed the largest negative zeta potential, due to the citrate anions adsorbed onto the surface of the nanoparticles. The ssAuNP showed the lowest absolute zeta potential, and thus confirmed that a surface density of 4 PEG/nm^2 was preferred in stabilizing the AuNP.

Table 1. Zeta potentials of AuNP solutions with various PEG surface densities.

PEG surface density (# PEG/nm^2)	0 (cAuNP)	1	4 (ssAuNP)	20
Zeta Potential (mV)	-33.2	-29.0	-10.0	-25.9

2.2.6. Radiographic properties – contrast measurements

The radiographic contrast of the AuNP solutions was determined using a GE Senographe 2000D mammography unit. The experimental setup is shown in Figure 7. A 12-well plate was fitted with a lead sheet that exposed adjacent wells in the plate below through two 1 cm (in diameter) holes. The plate was then positioned on the compression paddle, at a distance of 350 mm above the detector. For each condition presented below, the plate was imaged at energies ranging from 22 to 32 kVp, in 1 kVp increments.

Initially, 3 mL of DI-H_2O was placed in each of the two wells (left and right) and imaged at the full range of energies. The mAs at each kVp was tuned so that the average signal in each well containing DI-H_2O was 1500. These mAs values were then used for the remainder of the experiments. Next, the water in the right well was replaced with 3 mL of each of the following test solutions: ssAuNP (concentrated) and samples of gold chlorate that equated to solutions of 1, 3, and 6.5 mg Au/mL. C_L and C_R represent the signal intensities of the left and right well (respectively) when DI-H_2O was placed in both wells. SI_L and SI_R represent the signal intensities of their respective wells, when the test solution is present in the right well (Table 2).

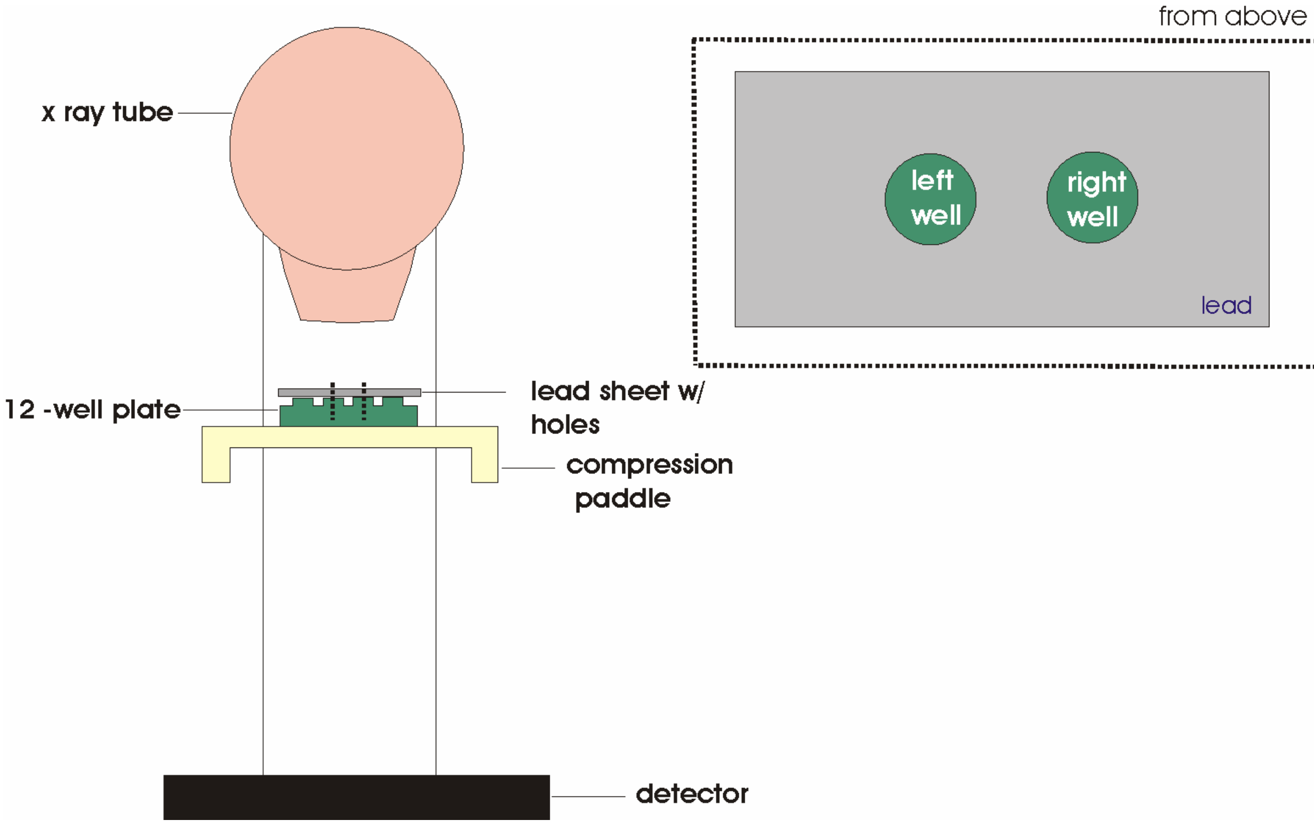

Figure 7. Experimental setup for radiographic contrast measurements. The solutions were placed in adjacent wells of a 12 well plate, which was placed underneath a lead sheet. The lead sheet has two 1 cm holes cut into it, to expose the two wells (left and right).

Table 2. Solutions placed in each of the two wells (left and right) for the calibration and experiment. The test solution is one of the following: ssAuNP (concentrated), or stock solutions of gold chlorate (1, 3, and 6.5 mg Au/mL).

	Left Well	Right Well
Calibration	DI-H_2O (C_L)	DI-H_2O (C_R)
Experiment	DI-H_2O (SI_L)	Test Solution (SI_R)

The metric *k*, defined as

$$k = \left| \frac{SI_R \times \left(C_L / SI_L \right) - C_R}{C_R} \right|,$$

was used to compare the contrasts of the various test solutions. The factor (C_L/SI_L) corrects for the time variation of the output of the mammography unit. *k* is tabulated for select energy values in Table 3.

Table 3. *k* tabulated at select energy values for the test solutions used.

	k			
kVp	ssAuNP (concentrated)	1 mg Au/mL	3 mg Au/mL	6.5 mg Au/mL
22	0.67	0.057	0.20	0.38
27	0.63	0.052	0.18	0.35
32	0.59	0.048	0.16	0.33

The concentrated ssAuNP offer a 67% (SD: 0.10) contrast compared to water, which is almost double that of the 6.5 mg Au/mL gold chlorate solution. Attempts to determine the *k*-value of more concentrated gold chlorate solutions resulted in precipitation of the salt. Although not shown here, the signal intensity of the ssAuNP (that were not concentrated) was found not to be statistically different from the signal intensity of the DI-H_2O. This makes the concentration of the ssAuNP an essential step in the development of AuNP as a radiographic contrast agent, a key innovation in this research period.

3. Key Research Accomplishments

I have successfully synthesized gold nanoparticles with a diameter of 21 (± 4.2) nm. The nanoparticles have been modified with PEG chains to make surface-stabilized AuNP (ssAuNP). I have characterized many of the structural and surface properties of the nanoparticles through TEM, UV/Vis spectroscopy, and zeta potential measurements. I have also, most importantly, managed to concentrate the AuNP solutions to a degree where they show observable radiographic contrast compared to water. An animal protocol covering the *in vivo* studies has been submitted and approved by the Institutional Animal Care and Use Committee (IACUC) here at the University of Pennsylvania. I am in the process of completing the necessary training and paperwork for procuring new animals and hope to begin the *in vivo* experiments in the coming month.

4. Reportable Outcomes

1) IACUC Protocol No: 802728. "Targeted gold nanoparticle contrast agent for digital breast tomosynthesis and computed tomography".

5. Conclusions

I have successfully synthesized colloidal AuNP that have been surface-stabilized in solution with PEG chains. The experience I have gained in synthesizing and sufficiently concentrating the ssAuNP can be used when I eventually attach the tumor-targeting ligand to the nanoparticles. Experimenting with ssAuNP in mouse tumor models will help me become familiar with the required techniques before I move on to the targeted AuNP.

Targeted AuNP could potentially serve as molecular agents for breast x ray imaging modalities such as mammography and digital breast tomosynthesis. These agents would be able to simultaneously provide structural and functional information on the tumor, and thus providing a more sensitive approach to early breast cancer detection.

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